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TITLE: Anti-Androgen Receptor RNA Enzyme as a Novel Therapeutic Agent for Prostate Cancer *in Vivo*

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14. ABSTRACT Prostate cancer is the second leading cause of cancer death among men in the western world. Androgen plays a crucial role in the development and growth of normal prostate gland and prostate cancer. Action of androgen is mediated by an androgen receptor (AR) and the AR exerts androgen-regulated gene expression. Standard therapy relies on androgen ablation to remove or block the action of androgens. This therapy results in a regression of the tumor because most primary tumor cells depend on androgens for growth. However, most prostate cancers eventually relapse as their tumors progress to androgen-refractory. Studies have indicated that the AR gene amplification and mutations are involved in androgen-refractory tumors. Therefore, blockage of the AR gene expression might provide a new approach to the management of the AR-dependent cancer. We have developed anti-AR RNA enzymes that are able to selectively and specially interact with the AR mRNA and cleave the AR mRNA in vitro. Unlike conventional chemotherapy, the enzymes would have lesser side effects because the compounds selectively destroy only the AR gene. This study proposed is to determine specific efficacy of these enzymes in vivo.					
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Introduction

Androgen is essential for the growth of both normal prostate gland and prostate cancer (CaP). Action of androgen is mediated by androgen receptor (AR), a member of the nuclear/steroid transcription factors. Although CaP is initially sensitive to androgen ablation therapy, the disease eventually progresses as androgen-refractory. The patients with the androgen-refractory cancers can no longer be cured by any type of conventional therapy. Although the molecular mechanisms of this disease progression remain unknown, studies have shown that AR gene amplification and mutations occur in androgen-refractory tumor specimens of almost all patients, but these evidences are rarely seen in initial CaP. Thus, AR gene amplification and mutations contribute to disease progress. We have initially developed short anti-AR RNA enzymes (about 20-40 bases) and these enzymes are able to specially bind to AR mRNA and efficiently catalyze the mRNA, resulting in inhibition of androgen-responsive gene expression *in vitro* and *in cultured cell systems*. The same result was further studied by another laboratory that these RNA enzymes catalyzed AR mRNA and decreased gene expression of prostate specific antigen (PSA) in human androgen-sensitive (LNCaP) and androgen-refractory (LNCaP-C4) cells, and inhibited growth and proliferation of these tumor cells. To test the efficacy of these enzymes on the prostate cancer *in vivo*, we firstly establish two mouse models for this study. The human androgen-sensitive and androgen-refractory tumor cells are microinjected into the prostate glands of the nude mice, respectively. In order to visualize the growth or metastasis of the tumor cells in the nude mice, we have transfected green fluorescent protein (GFP) vector into the two tumor cells. GFP is expressed in both androgen-sensitive and androgen-refractory cells. We selected the GFP as a reporter marker because it is used as a detection tool for cell sorting, measurement of tumor growth in living cells or tissues and it is not toxic to host cells. Secondly, we have synthesized and delivered these anti-AR RNAs into these tumor cells using different methods. Dynamic interaction of the enzymes with the targeted tumor cells in intracellular movement were observed by the fluorescent imaging system. We will also test effects of these enzymes on the tumor growth in the androgen-sensitive and androgen-refractory tumor cells grown as xenografts in the nude mice compared to that of the control group. At different time points, the tumor sizes in the treated and control groups have be measured. Expression levels of AR and PSA from tissue samples have be detected after the animals were sacrificed. Our study showed that although anti-AR RNAs were able to inhibit AR and its related gene expression in cell system, *in vivo* these enzymes failed to repress AR expression and tumor growth compared to the control group because there are no efficient deliver systems to tumor cells.

Body

Androgen is the most important endocrine factor regulating the development and growth of prostate gland and the action of androgens is mediated by the androgen receptor (AR), which is a ligand-responsive transcription factor (1). The AR exerts its action by binding to the androgen response element, to control expression of androgen-regulated genes (2). Overexpression of the AR in mouse prostate gland induced aged-AR transgenic mice to develop intraepithelial dysplasias similar to human prostate cancer (3). Initial prostate cancer is effectively treated with androgen ablation. However, tumor eventually becomes refractory to anti-androgen treatment (4, 5). Although the molecular events of progression from androgen-sensitive to androgen-refractory tumor are unclear, several studies have showed that this progress is associated with AR gene amplification and mutations (6-8). Our study has shown that high expression of AR was observed in human prostate specimens compared to the normal prostate tissues adjacent to the cancers (**Fig.1**).

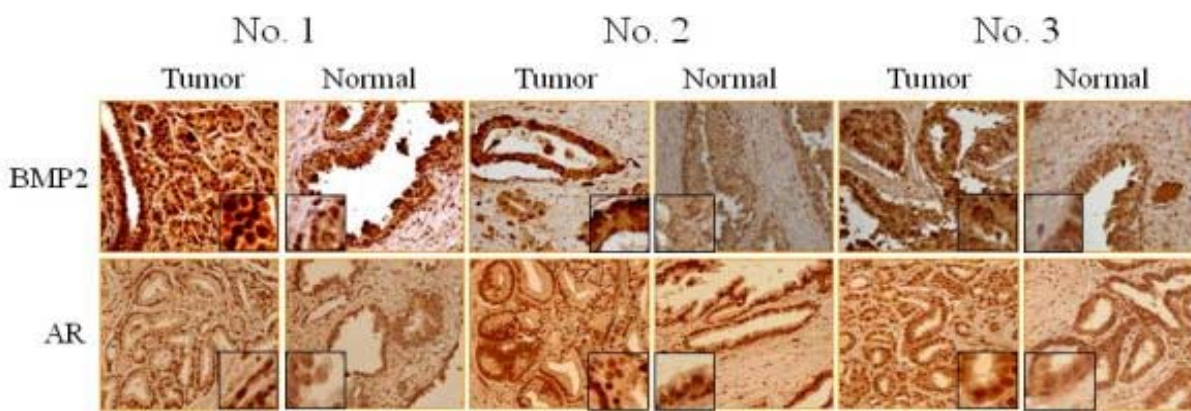


Fig 1. Immunohistochemical analysis of BMP2 and AR in normal and neoplastic prostatic samples. Deparaffinized sections of prostate adenocarcinoma and normal prostate tissues adjacent to the tumors were incubated with antibodies specific for BMP2 and AR respectively. The sections were treated with biotinylated secondary antibody and subsequently streptavidin-biotin peroxidase. The signal was detected by 3,3'-diaminobezidine and the tissues were counterstained with hematoxylin. The tissues sections were photographed with an AxioCam MR-MRGrab camera imaging system (Carl Zeiss Vision, Munich, Germany). Among 137 samples, 9 pairs of the tumors contain normal tissues adjacent to the same cancers. These patient ages were between 58 and 73 years old. Prostate specific antigen (PSA) ranged from 7 to 60.4 ng/ml. Immunohistochemistry study showed that expression of BMP2 and AR was more intense in these cancers than the normal tissues. Higher expression levels of BMP2 and AR were also found within the nucleus in these tumors.

Current therapies are not effective in the control of androgen-refractory prostate cancer (9). Inhibition of AR expression in the tumor cells may provide a way to control disease progress. We initially developed small anti-AR RNA enzymes that are able to specifically and efficiently catalyze AR messenger RNA *in vitro* (10). Based on these results, we have studied whether these anti-AR RNA enzymes have biological functions *in vivo* in this proposal. At first, we have established two types of human prostate cancer cell lines, androgen-sensitive (LNCaP) and androgen-refractory (LNCaP-C4), that express green fluorescent protein (GFP) (**Fig 2**).

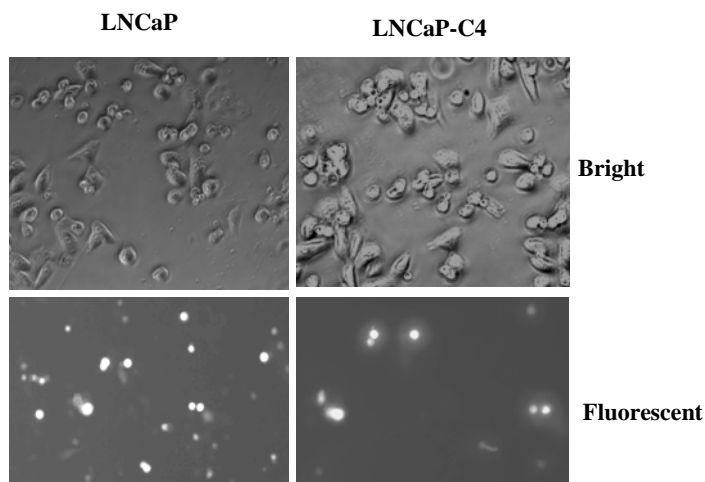
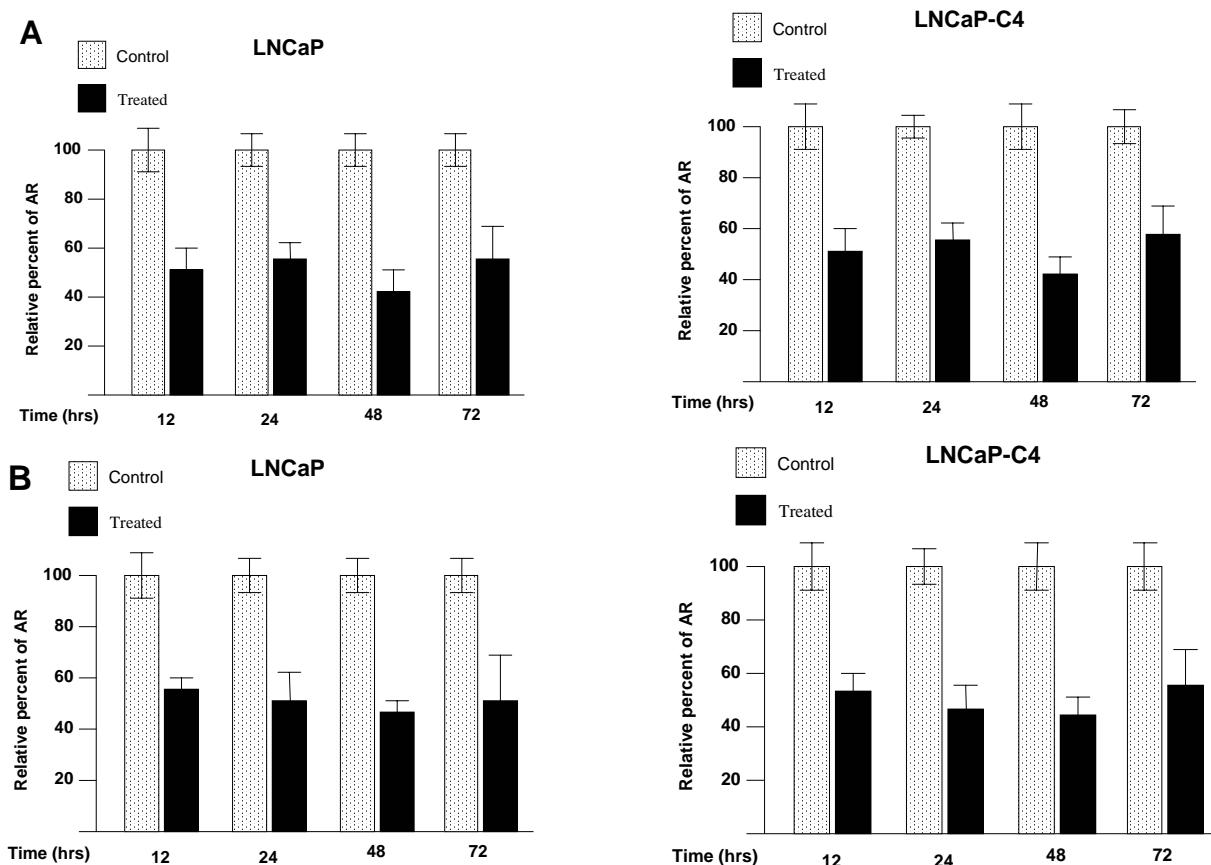
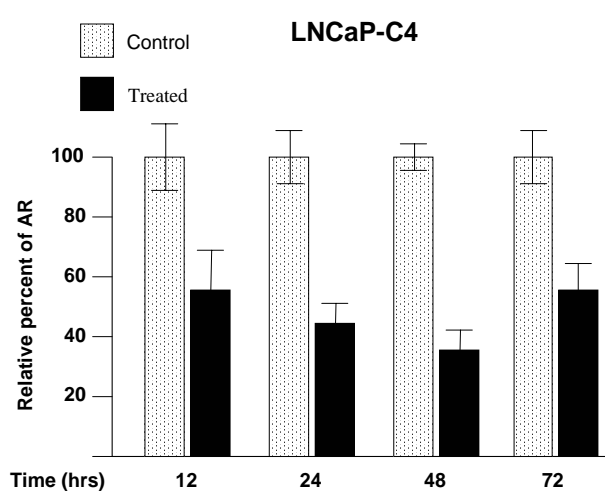
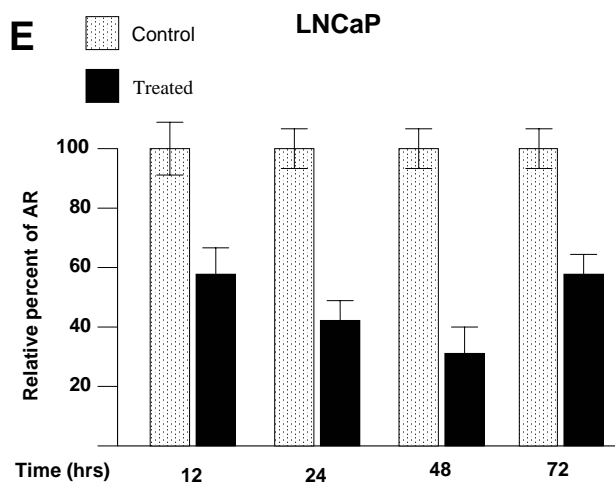
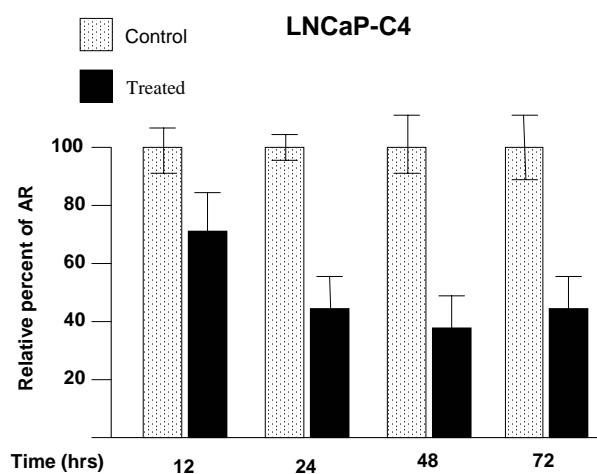
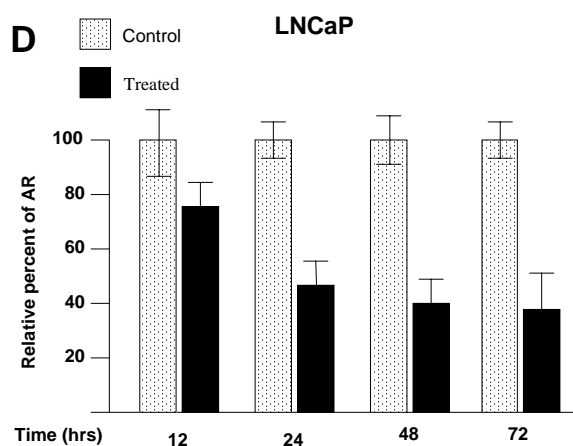
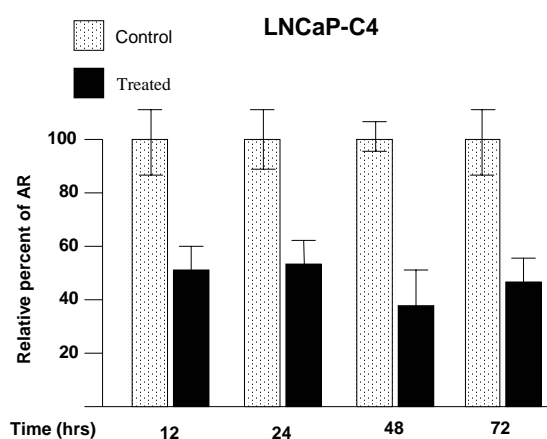
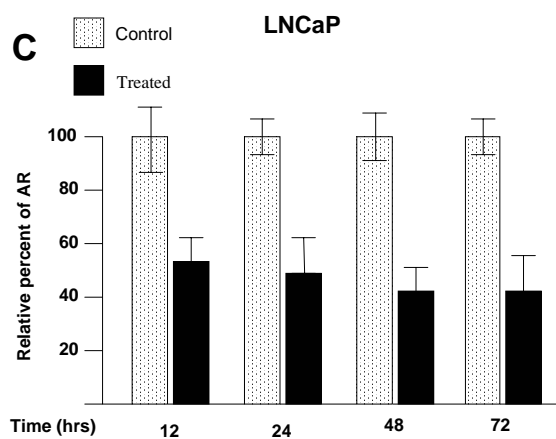


Fig. 2. Expression of GFP in LNCaP and LNCaP-C4 cells. Magnification x 10

Secondly, we have detected biological effects of these RNA enzymes on endogenous expression of AR and AR-regulated genes; prostate specific antigen (PSA) and kallikrein 4 (KLK4) at different time points in these two transfected GFP cancer cell lines using quantitative real time PCR (qRT-PCR). These results showed that four different RNA enzymes were able to inhibit expression of endogenous AR gene in the two cell lines (**Fig. 3A-D**). The highest effect of these enzymes was at 48 hours after transfection. Furthermore, multiple RNA enzymes had higher effects on inhibiting expression of AR compared to the individual anti-AR-enzyme (**Fig. 3E**). Biological effects of these RNA enzymes had dose-dependent at 24-, 48- and 72-hour time periods (**Fig. 3F**). Also, we further analyzed AR-regulated genes, PSA and KLK4. **Fig. 4** and **5** showed the same results with AR genes. These results were represented at 13th Annual Cancer Research Symposium, November, 2004, San Antonio, Texas (see appendix).





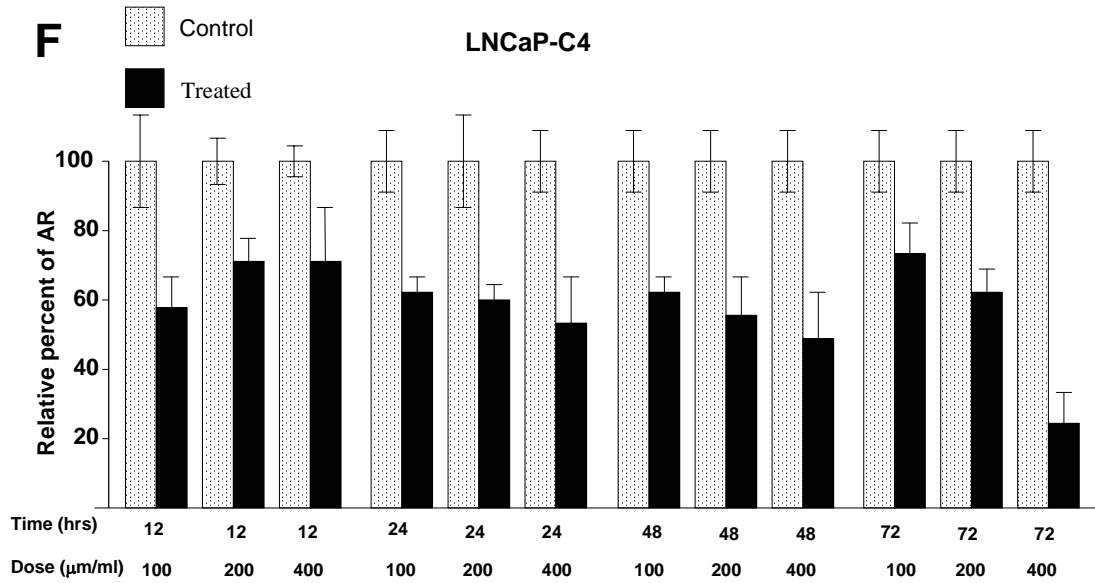
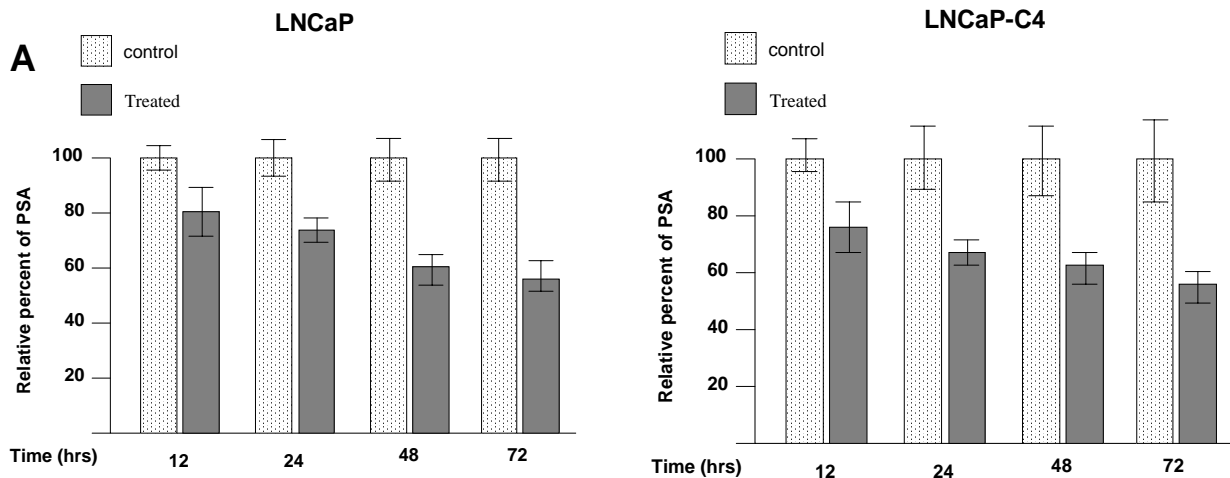
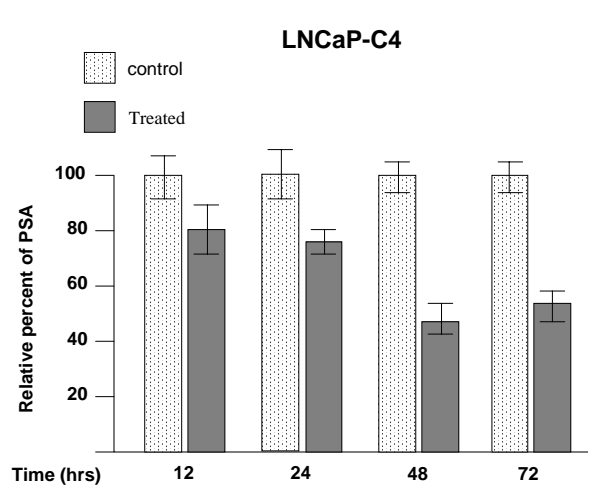
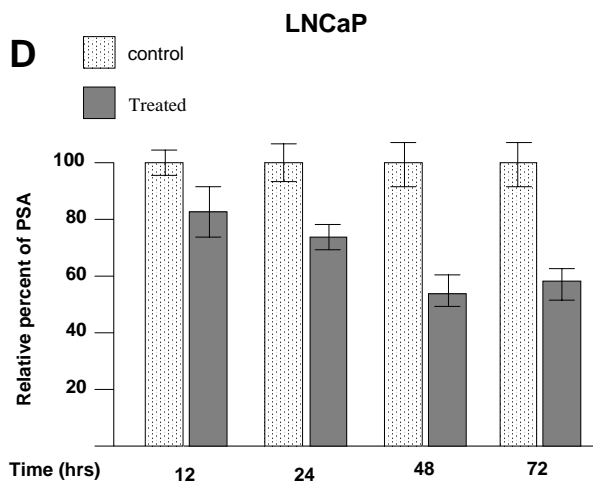
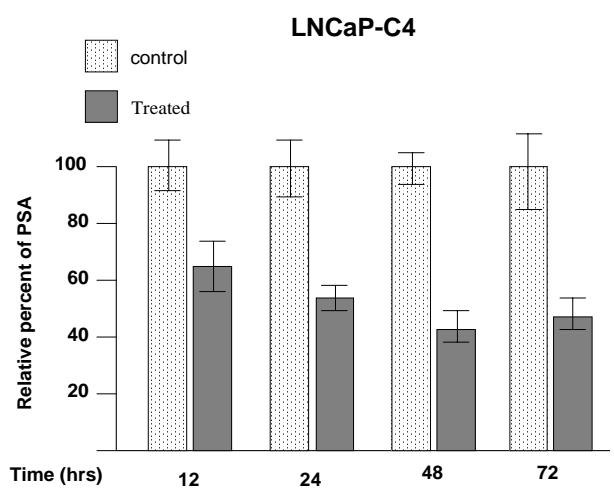
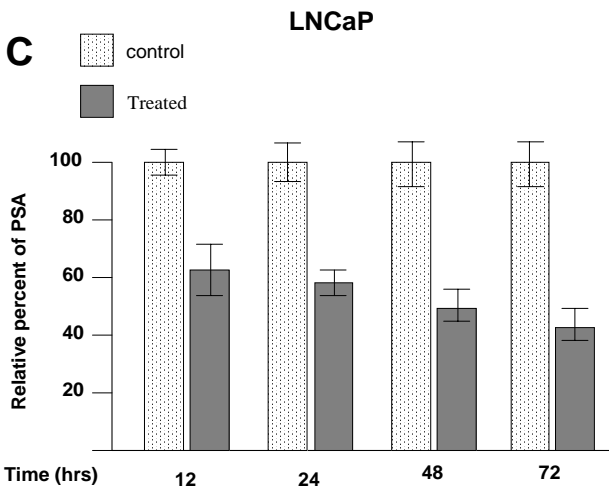
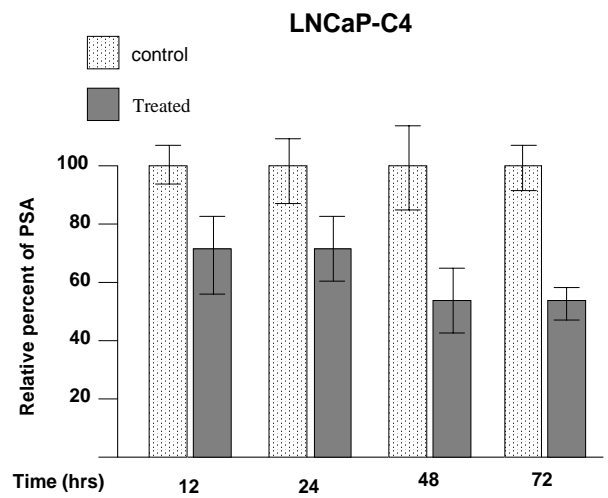
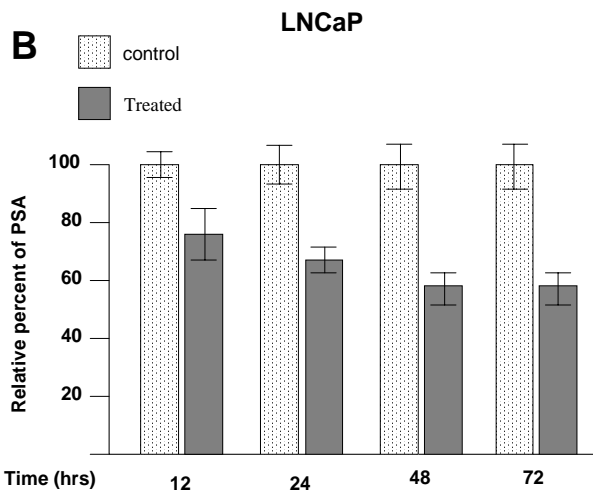


Fig 3. Biological effects of anti-AR enzymes on AR gene expression in human prostate cancer cells. 100 μM of individual or multiple anti-AR RNA enzymes or negative control (Ambion, Austin, TX) was transfected into the two human prostate cancer cells, respectively, according to the protocol provided by the manufacture (Ambion). After different time points, total RNA was isolated and cDNA was reversely transcribed. 100 ng of the transcribed cDNA were used for quantitative real time PCR. Amplification reactions were analyzed in real-time on an ABI 7500 (Applied Biosystems, Foster City, CA) using SYBR Green chemistry and the threshold values were calculated using SDS2 software (Applied Biosystems). Thermal cycling parameters were 95°C for 30s and 60°C for 1 min, 40 cycles. Reactions were performed in quadruplicate and threshold cycle numbers were averaged. A single melt curve peak was observed for each sample used in data analysis, confirming the purity and specificity of all amplified products. The threshold data generated was normalized to cyclophilin A. Primers used for AR and cyclophilin gene amplifications were as follows: cyclophilin; forward 5' -ggtgacttcacacgccataa- 3'. reverse 5' -catggcctccacaatattca- 3'; AR: forward 5' -ggccaggaaagcgacttcac- 3' reversed 5'-gacacaagtgggactgggatagg- 3'. A, anti-AR RNA enzyme 1; B, anti-AR RNA enzyme 2; C, anti-AR RNA enzyme 3; D, anti-AR RNA enzyme 4; E, four anti-AR enzyme combination; F, different doses of anti-AR enzyme 3.





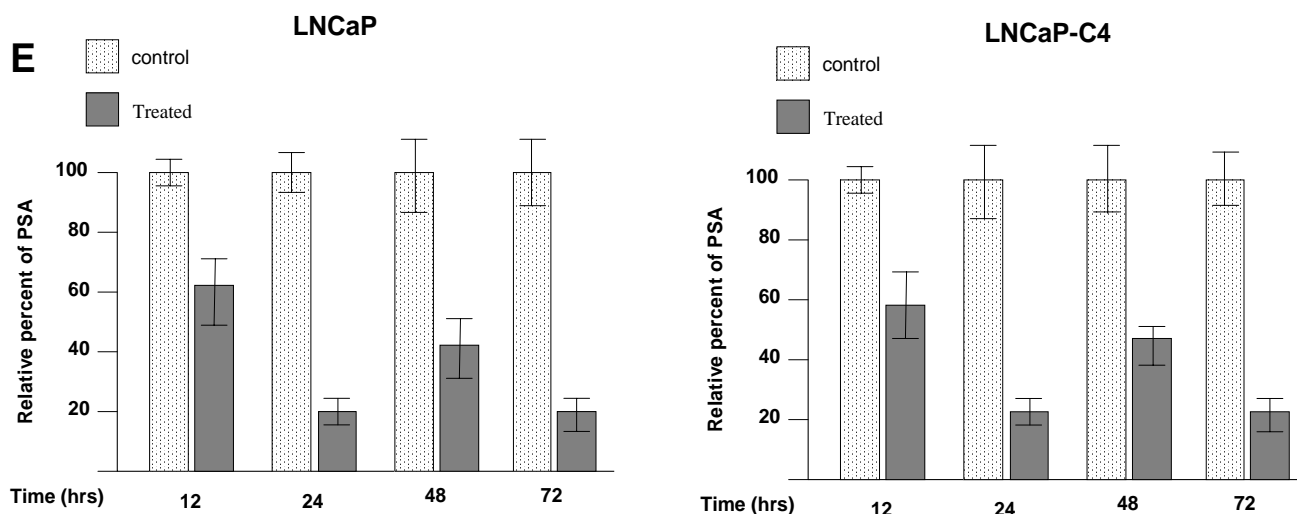
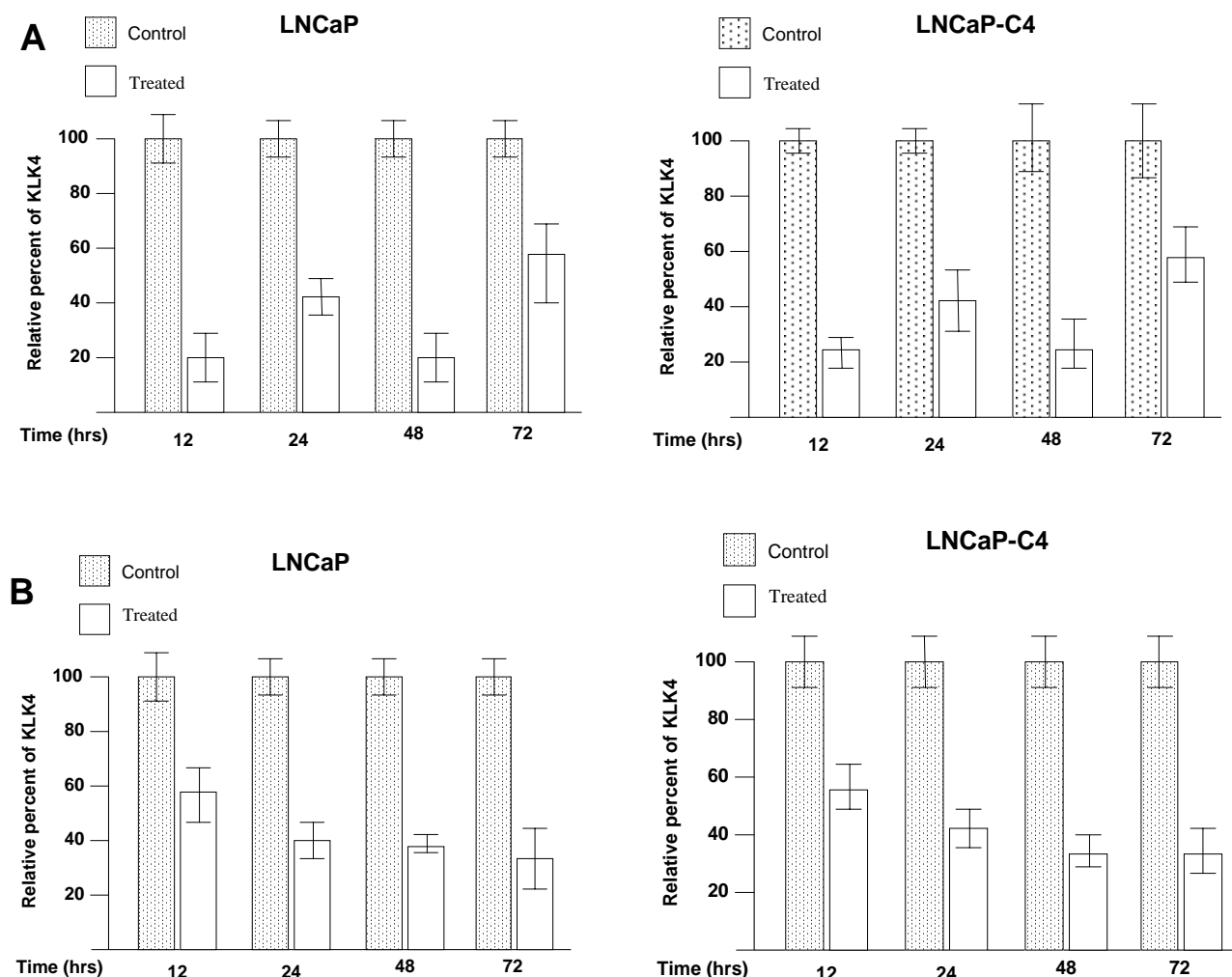


Fig 4 Biological functions of anti-AR enzymes on PSA gene expression in human prostate cancer cells. Experimental procedures were described above in Fig 3. Primers used for PSA gene amplification were as follows: forward 5'-gagccaaggaggagggtctt-3', reverse 5'-tcccccatagtgaatcagctt-3'. A, anti-AR RNA enzyme 1; B, anti-AR RNA enzyme 2; C, anti-AR RNA enzyme 3; D, anti-AR RNA enzyme 4; E, four anti-AR enzyme combination.



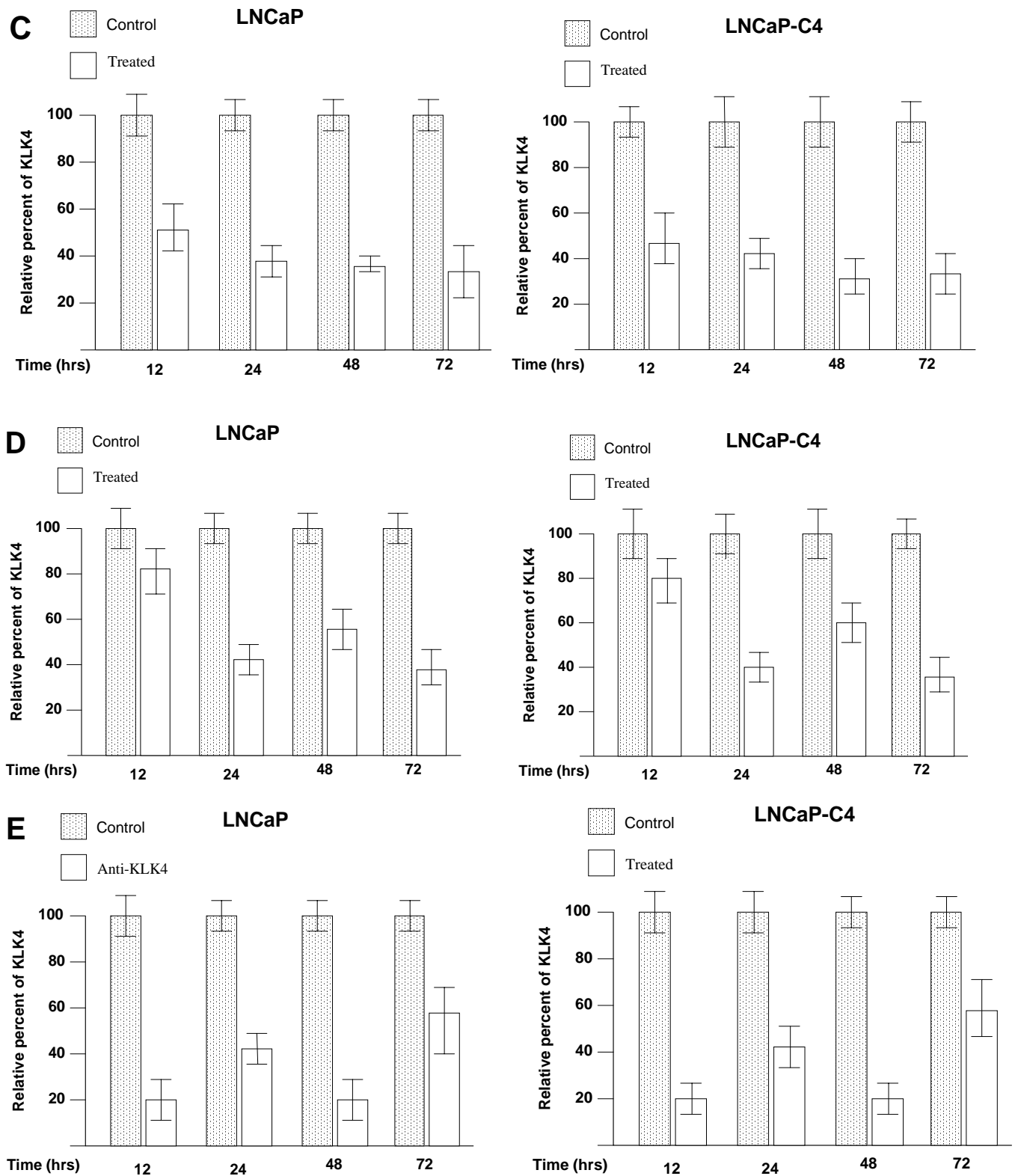


Fig 5 Biological functions of anti-AR enzymes on KLK4 gene expression in human prostate cancer cells. Experimental procedures were described above in Fig 3. Primers used for KLK4 gene amplification were as follows: forward 5' –cagacccttgctcgctaacg- 3', reverse 5' –gctccggatgggtgcagact- 3'. A, anti-AR RNA enzyme 1; B, anti-AR RNA enzyme 2; C, anti-AR RNA enzyme 3; D, anti-AR RNA enzyme 4; E, four anti-AR enzyme combination.

In addition, two anti-AR DNA and negative control oligonucleotides were subcloned into lentiviral vector (**Fig. 6 A, B**). The siRNA-expressing vector transiently transfected into LNCaP cells, and high efficiency of GFP expression as a marker of siRNA deliver was seen in the tumor cells 24 and 48 hours after transfection (**Fig. 6C**). The AR-siRNAs driven by lentiviral vector were able to inhibit expression of AR and PSA genes at 24, 48 and 72 hours after transfection (**Fig. 6D-F**).

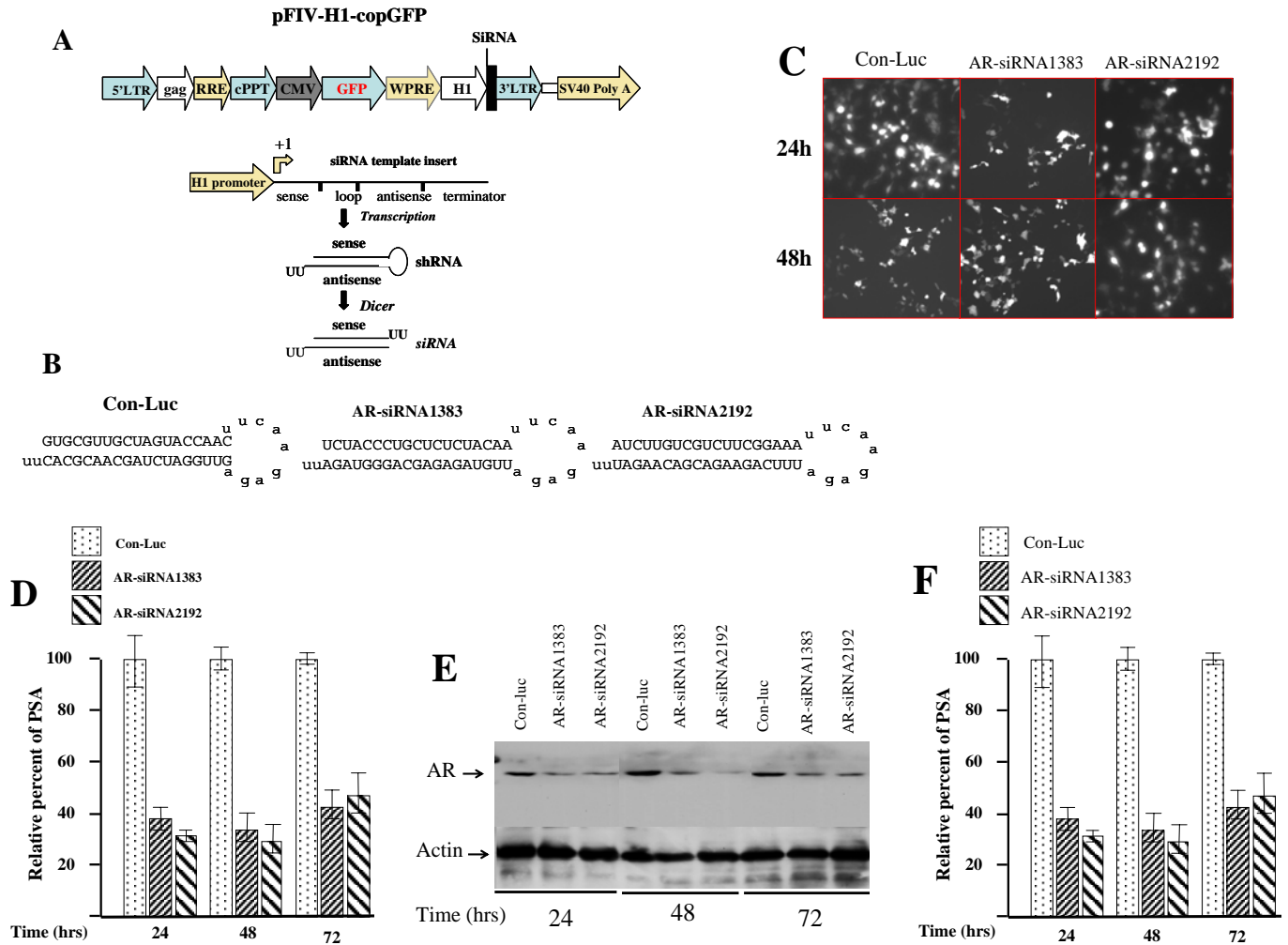


Fig 6. Inhibition of AR by siRNA-expressing lentiviral vector *in vitro*. (A) Schematic diagram of the siRNA-expressing lentiviral vector. The short hairpin form of siRNA is expressed under the control of a human H1-RNA Pol III promoter. The vector also contains CMV promoter driving the green fluorescent protein (GFP) marker gene for tracking transduced cells. (B) Predicted secondary structure of the siRNA targeting human ARs (AR-siRNA1383, AR-siRNA2192) and control siRNA against luciferase gene (con-luc). (C) Deliver of the siRNA-expressing lentiviral vector to LNCaP cells. siRNA expression cassettes encoding a con-luc (negative) or AR-siRNAs (AR-siRNA1383, AR-siRNA2192) were transiently transfected into LNCaP cells by using siPORT™ Amine Transfection Agent (Ambion). Twenty-four and 48 h after transfection, GFP expression in the cells were seen under fluorescent microscope. (D) Repression of AR expression in LNCaP cells by AR-siRNA-expressing lentiviral vectors. Individual siRNA lentiviral vectors including con-luc, AR-siRNA1383, AR-siRNA2192 were transfected into LNCaP cells, respectively according to the protocol provided by the manufacture

(Ambion). After different time points, total RNA was isolated and cDNA was reversely transcribed. 100 ng of the transcribed cDNA were used for qRT-PCR. Amplification reactions were analyzed in real-time on an ABI 7500 (Applied Biosystems, Foster City, CA) was described above. **(E)** Detection of AR expression in the treated and control groups by Western blot assay. **(F)** Expression of PSA gene in the control and treated groups by qRT-PCR assay.

For *in vivo* study, synthesized AR-siRNAs and control siRNA by using the tail vein injection were delivered to immunodeficient mice with expression of LNCaP cells in prostate gland 3 weeks after orthotopic implantation. Injection was repeated every two days and mice were sacrificed at 7 and 14 days after siRNA treatment. The results showed that there are no significant differences of tumor size between control and AR-siRNA treatment groups at two time points (data not shown). Also, expression of AR and PSA genes from the control and treated groups showed no statistic difference (**Fig. 7**).

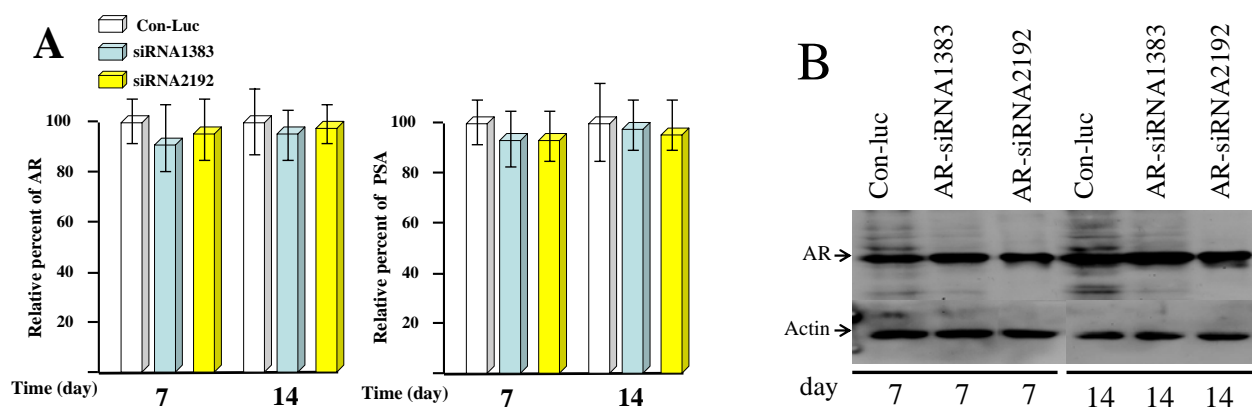


Fig 7. Effect of AR siRNA *in vivo*. Immunodeficient mice (n = 4 mice/per group) were purchase from Taconic, Germantown, NY and anesthetized with cocktail (Sigma). A low middle incision was made, and fluorescent LNCaP cells (4×10^5 cells/10 μ l) in RPML 1640 medium were implanted into the ventral prostate lobes using a 30-gauge needle with a 0.1-ml syringe. The abdominal wall was sutured closely. Synthesized two siRNAs (AR-siRNA1383, AR-siRNA2192) and one control siRNA (con-luc) were purchased from Dharmacon Inc. (Lafayette, CO) and delivered *in vivo* using a modified “hydrodynamic” transfection method (11) by which 30 μ g siRNA dissolved in 1 ml PBS was rapidly injected into the tail vein 3 weeks after orthotopic implantation. The injection was repeated every two days. Mice were sacrificed at 1 and 2 weeks after treatment. **(A)** Expression of AR and PSA genes in the control and treated group by qRT-PCR. The qRT-PCR method was described above. **(B)** Expression of AR protein in the treated and control groups by Western blot analysis.

Key research accomplishments

1. 137 human prostate normal and cancer specimens were detected by immunohistochemistry assay using anti-BMP2 and AR antibodies.
2. Two FGP prostate cancer cell lines were established.
3. Four designed anti-AR RNA enzymes were able to inhibit endogenous AR and AR-regulated gene expressions in two human prostate cell lines.

4. AR siRNA-driven by lentirival vector had more effective on repressing AR and AR-regulated gene expression in LNCaP cells.
5. The synthesized AR siRNA was not able to inhibit AR and its related gene expression in mouse model.

Reportable Outcomes

In this project, AR expression in human prostate normal and cancer specimens were observed by immunohistochemistry and the results showed that AR is highly expressed in prostate tumor tissues compared to the normal samples. Secondly, four anti-AR RNA enzymes were designed and synthesized. The four anti-AR RNA enzymes were able to inhibit expression of AR and AR-related genes, PSA and KLK4 in cell cultured system. In addition, lentirival vectors with expressing AR siRNAs had more effective on inhibition of AR gene expression in human prostate cancer cells, LNCap and LNCaP-C4. However, synthesized AR siRNAs had not able to inhibit AR and PSA gene expression *in vivo*.

Conclusions

In this report, we demonstrated that designed anti-AR RNA enzymes are capable of inhibiting expression of AR, PAS and KLK4 genes in two human prostate cells *in vitro*. But there are not significances *in vivo*. It is required for high concentration of siRNA that targets to its targeted tissue by high efficiency of deliver system.

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Regulation of Androgen Receptor Expression in Human Prostate Cancer Cells by a Novel Transcription Factor and Small Interference RNA. *Chen S, Wu, Y, Chuang, H, MacDougall M. The University of Texas Health Science Center at San Antonio, TX 78229, San Antonio, TX.

Androgen Receptor (AR) functions as a ligand-activated transcription factor for androgen-regulated genes. AR amplification and mutations play the critical roles in recurrence and metastasis of certain prostate cancers. **Objective:** First to investigate regulatory elements of AR gene in human prostate cancer versus normal cells; second to examine small interference RNA (siRNA) specific for AR gene in inhibiting AR expression in human cancer cells. **Methods:** Various constructs of human AR promoter-luciferase reporter gene were transfected into human prostate cancer (LN-cap) and normal (RWPE) cells, and luciferase activity was analyzed. A critical responsible element was identified and binding of nuclear protein(s) to this element was examined by electrophoretic mobility shift assay (EMSA). In addition, inhibition of AR expression by the siRNA was determined by quantitative real time PCR and Western blotting assays. **Results:** We characterized that one element in human AR promoter was responsible for AR gene expression. Mutations of this element resulted in a 2- and 3-fold decline of promoter activity in RWPE and LN-cap cells. A series of mutation and competition assays showed that the element interacts with a novel nuclear protein by EMSA and expression levels of this nuclear protein was higher in the cancer cells than that of normal cells. The consensus sequence of this element is conserved across species. In addition, the siRNA had effects on inhibiting AR gene expression in LN-cap cells and the highest efficiency at 12 hours after treatment. **Conclusions:** This study speculates that this element in AR gene promoter plays some distinct functions in regulating AR expression in human cancer versus normal cells. Furthermore, the siRNA provides a new avenue for inhibition of androgen action by selective mRNA degradation with its potential therapeutic application through targeted gene delivery vectors. This work was supported by DOD grant W81XWH and NIDR grant DE11658.

The abstract was accepted by The IMPaCT meeting and will be present on September 8, 2007, Atlanta, Georgia.

Bone morphogenetic proteins 2 induced androgen receptor expression via extracellular signal-regulated protein kinases and heterogeneous nuclear ribonucleoprotein K signal pathways in human prostate cancer progression

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Androgen receptor (AR) is essential for the growth of the prostate cancer cells and involved in tumor progression from androgen dependent to androgen refractory stages. Androgen refractory cancer cells frequently metastasize to bone and cancer-skeletal metastasis is often characterized as osteoblastic; suggesting that bone microenvironment is suitable to cancer growth and cancer cells further promote bone cell proliferation and differentiation. Bone morphogenetic proteins (BMPs) are mediators of skeletal formation. Prostate cancer cells produce a variety of BMPs such as BMP2. The mechanisms underlying the relationship between BMP2 and AR-signaling pathways in progression of prostate cancer have not completely understood. Here we report that AR expression is induced by BMP2 through extracellular signal-regulated protein kinases (ERK^{42/44})/heterogeneous nuclear ribonucleoprotein K (hnRNP K) signal pathways in human prostate cancer cells (LNCaP-C4). Western blot assay showed that BMP2 dramatically increased AR expression in LNCaP-C4 cells. Promoter functional analysis by gradually deleted fragments of the human AR promoter demonstrated that a BMP2-response element is located in the AR proximal regulatory region. By electrophoretic mobility shift assay, protein purification and Mass spectrometry, we identified a protein, hnRNP K that binds to the BMP2-responsible element by using antibody, oligo competition and chromatin immunoprecipitation assays. BMP2 elevated hnRNP K accumulation into nucleus and increased recruitment of hnRNP K to its target site in the AR promoter in vivo. Furthermore, high expression levels of hnRNP K and AR within nucleus were measured in human prostate cancer tissues (n=50) as compared to the normal prostate samples adjacent the same cancer specimens by immunohistochemistry. Plasma levels of prostate specific antigen (PSA) from these cancer patients were ranged from 8.0 to 98 nanogram/ml. We further found that BMP2 induced the translocation of hnRNP K into nucleus via phosphorylation of kinase ERK^{42/44}, indicating that BMP2 stimulates AR expression via ERK^{42/44}/hnRNP K signal pathways. Second, we observed that BMP2 enhanced expression of dentin matrix protein1 (DMP1) gene in LNCaP-C4 cells. DMP1 is one of extracellular matrix proteins important for bone development and formation. Increased expression of DMP1 was detected in human prostate cancer tissues that metastasized to bone. Potential AR binding sites were found in human DMP1 promoter. This result indicates that prostate cancer cells preferentially metastasize to bone as BMP2 induces AR expression by ERK^{42/44}/hnRNP K signaling pathways, promoting the growth of prostate cancer cells in bone marrow microenvironment and AR further increases DMP1 gene transcription resulting in proliferation and differentiation of bone marrow cells. These signals between epithelial

(prostate cancer) and mesenchymal (bone marrow) cells mutually regulate the growth of prostate and osteoblast cells. IMPACT: This project provides a novel mechanism that BMP2 stimulates AR expression by ERK^{42/44} /hnRNP K signal pathway in prostate cancer progression and might have a new clue for diagnosis and treatment of the human prostate cancer especially in the advanced stage. High expression of hnRNP K and DMP1 could provide biomarkers for prostate cancer diagnosis in particular bone metastasis. This study was supported by DOD grant W81XWH-04-10253.